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REVIEW ARTICLE

Lifestyle, metabolism and environmental adaptation in *Lactococcus lactis*

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One sentence summary: The lifestyles of *Lactococcus lactis* in different environmental niches and their adaptation under dynamic conditions.

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ABSTRACT

Lactococcus lactis serves as a paradigm organism for the lactic acid bacteria (LAB). Extensive research into the molecular biology, metabolism and physiology of several model strains of this species has been fundamental for our understanding of the LAB. Genomic studies have provided new insights into the species *L. lactis*, including the resolution of the genetic basis of its subspecies division, as well as the control mechanisms involved in the fine-tuning of growth rate and energy metabolism. In addition, it has enabled novel approaches to study lactococcal lifestyle adaptations to the dairy application environment, including its adjustment to near-zero growth rates that are particularly relevant in the context of cheese ripening. This review highlights various insights in these areas and exemplifies the strength of combining experimental evolution with functional genomics and bacterial physiology research to expand our fundamental understanding of the *L. lactis* lifestyle under different environmental conditions.

Keywords: lactic acid bacteria; *Lactococcus lactis*; metabolism; adaptation; environmental fitness; dairy application

INTRODUCTION: *LACTOCOCCUS LACTIS* A PARADIGM LAB SPECIES

Lactococcus lactis plays an important role in the fermentation industry where it is applied in the production of a range of fermented products. Due to its importance in industrial food fermentations, *L. lactis* has been the subject of fundamental research for over a century. Initially described as *Bacterium lactis*, it was first reclassified as *Streptococcus lactis* and later as *Lactococcus lactis* (van Hylckama Vlieg *et al.* 2006). Many of the *L. lactis* applications in food fermentation are within the dairy sector, using milk as the raw food material that is converted by lactococcal fermentation to a range of products in different regions of the world (for a review, see Cavanagh, Fitzgerald and McAuliffe 2015). The primary role of lactococci in these fermentations, acidification, is dependent on efficient conversion of the milk-sugar lactose to lactic acid, which contributes

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to the extended shelf life of fermented milk products by preventing the outgrowth of pathogenic and spoilage organisms. Additionally, *L. lactis* contributes to the texture and flavour characteristics of the fermented products. Texture properties of fermented milk products are not only dependent on the changes in physical chemical properties of the milk proteins that are elicited by the fermentative acidification but also influenced by the lactococcal production of extracellular polysaccharides that contribute to the rheological properties of fermented milk products (for a review, see Kleerebezem *et al.* 1999; Zeidan *et al.* 2017). The importance of flavour formation by *L. lactis* is especially prominent in a variety of cheeses of which the production includes a long ripening period during which milk constituents are converted to flavour compounds that determine the taste of the cheese. The lactococci play a central role in flavour formation by the degradation of milk proteins and the conversion of the resulting amino acids into volatile aroma components. Extracellular proteolysis, peptide and amino acid import and intracellular peptidolysis by peptidases are of obvious importance in this process, and the proteins involved as well as the regulation of their expression have extensively been studied in *L. lactis* (for reviews, see Kunji *et al.* 1996; Savijoki, Ingmer and Varmanen 2006; Kok *et al.* 2017). Nevertheless, recent studies underpin that our understanding of amino acid import can still be refined. This is exemplified by the recent discovery of the co-existence of two isogenic *L. lactis* subpopulations that depend on either high- or low-affinity import systems of the essential amino acid methionine, which is controlled by a riboswitch present in the promoter region of the genes encoding the high-affinity importer (Hernandez-Valdes, van Gestel and Kuipers 2020). Similarly, the transport-coordinating role of the peculiar presence of two distinct and competing substrate binding domains in the *L. lactis* GlnPQ importer for asparagine, glutamine and glutamic acid was only recently unravelled (Gouridis *et al.* 2015; Fulyani *et al.* 2016; Schuurman-Wolters *et al.* 2018). Amino acid catabolism by *L. lactis* plays an essential role in flavour formation and although the metabolic activities that are linked to flavour formation are not fully deciphered, a number of pathways and key enzymes involved in this process have been described (for a review, see Smid and Kleerebezem 2014).

For a long time, *L. lactis* has served as a paradigm organism in research of the lactic acid bacteria (LAB), where it contributed tremendously to our present understanding of the molecular biology, genetics, gene regulation and metabolism of this group of bacteria (for reviews, see Mahony *et al.* 2016; Papadimitriou *et al.* 2016; Kok *et al.* 2017; Price *et al.* 2019). Especially since the 1970s, the research on *L. lactis* genetics and the introduction of genetic engineering have contributed to our molecular insight in key functions of *L. lactis* in dairy fermentations, serving as an example and source of inspiration for similar research in a variety of other LAB. This exemplary role of *L. lactis* research is underpinned by many common strategies, procedures and tools that are used in genetic engineering of LAB that were pioneered in *L. lactis* (for reviews, see Mills 2001; Daniel *et al.* 2011; Landete 2017; Bron *et al.* 2019). In the last couple of decades, the scientific investigations of *L. lactis* have progressed to embrace the wealth of the post-genomic approaches. Simultaneously, post-genomic research of other LAB species also accelerated, leading to an explosion of information related to the genetics, diversity and evolution of the LAB in general. To some extent, this development has diminished the status of *L. lactis* as paradigm species, although it is still among the most intensely investigated LAB. Below, we will discuss a selection of comparative genomics-derived insights in the species *L. lactis*, including the resolution of the genomic basis of its subspecies division, the understanding of lactococcal metabolism through metabolic and genetic engineering and genome-scale metabolic models, and the investigations of its capacity to adjust its lifestyle, including the use of experimental evolution approaches. Finally, we will discuss our current knowledge of the adaptation of *L. lactis* to dairy application environments, including its adjustment during dormancy and near-zero growth rates that are particularly relevant in the context of cheese ripening.

LACTOCOCCUS LACTIS SUBSPECIES AND GENOMIC REFLECTIONS OF NICHE ADAPTATION

Within the species *L. lactis*, the two subspecies (spp.) *lactis* and *cremoris* are considered the most important in biotechnological applications. On basis of the sequence of their 16S rRNA encoding gene, these subspecies were estimated to have diverged ~17 million years ago (Bolotin *et al.* 2004a) and are traditionally discriminated by subspecies-specific phenotypic traits (Schleifer *et al.* 1985; Kim, Ren and Dunn 1999; van Hylckama Vlieg *et al.* 2006; Fernandez *et al.* 2011). Strains belonging to the subspecies *lactis* can produce ammonia from arginine, utilise maltose and grow at 40°C and in the presence of 4% sodium chloride, whereas strains of ssp. *cremoris* lack these properties. Several studies have described the distinction of *L. lactis* genotype lineages that were also designated *cremoris* and *lactis*, which can be discriminated by various genetic typing methods like multi-locus sequence typing, lineage-specific PCR and high-resolution genetic fingerprinting (Beimfohr, Ludwig and Schleifer 1997; Pu *et al.* 2002; Kutahya *et al.* 2011; Laroute *et al.* 2017). However, although the names '*cremoris*' and '*lactis*' are used for both genetic and phenotypic discrimination of the *L. lactis* strains, these classifications are not entirely congruent because several strains that belong to the *cremoris* genetic lineage display a subspecies *lactis* phenotype (van Hylckama Vlieg *et al.* 2006; Kelly, Ward and Leahy 2010; Parapouli *et al.* 2013; Cavanagh, Fitzgerald and McAuliffe 2015). Despite various attempts, including detailed analysis of selected genes and comparative genomics, the genetic basis of the *lactis* and *cremoris* phenotype–genotype disparity was only recently resolved. The phylogenetic analysis of single nucleotide polymorphisms in the core genome of 43 *L. lactis* strains indicated that strains displaying the incongruent *lactis* phenotype and *cremoris* genotype share a common ancestor with *L. lactis* subspecies *lactis* and that strains with a congruent *cremoris* genotype and phenotype evolved only once (Wels *et al.* 2019). The genetic repertoire that allowed the resolution of the *lactis-cremoris* phenotype–genotype discrepancy encoded not only a variety of hypothetical proteins, transporters and regulators of unknown specificity but also several functions associated with heat and osmotic stress response that may explain the corresponding differences in growth at elevated temperature and salt concentrations. Moreover, erosion of the arginine deiminase pathway was associated with strains displaying the spp. *cremoris* phenotype, whereas redundancy of the maltose utilisation gene cluster was found in strains with the spp. *lactis* phenotype (Wels *et al.* 2019). Interestingly, whereas the vast majority of phenotype *cremoris*, genotype *cremoris* strains are found in dairy environments, the strains displaying the ssp. *lactis* phenotype are isolated from both dairy and non-dairy environments, in particular from plant materials (Kelly, Ward and Leahy 2010).

Two dairy-derived model strains have been used extensively for fundamental research in *L. lactis*. Strain IL1403 is a plasmid

free derivative of IL594, which was originally isolated from a cheese starter culture (Bolotin *et al*. 2001). One of the plasmids of IL594 allows it to metabolise citrate, which classifies this strain as a *L. lactis* subspecies *lactis* biovar *diacetylactis*. Strain MG1363 was generated by curing of prophages and plasmids from strain NCDO712, a strain isolated from a dairy starter culture (Gasson 1983; Wegmann *et al*. 2007). It has a subspecies *cremoris*-like genotype and a subspecies *lactis*-like phenotype. While plasmid and prophage curation of these strains was an important step to enable genetic engineering using plasmid-based approaches, the fact that IL594 and NCDO712, respectively, harboured 7 and 6 plasmids (Gorecki *et al*. 2011; Tarazanova *et al*. 2016) hints at their importance in the habitual environment of these strains. This was further corroborated by the observation that in both strains several plasmid encoded functions are critical for efficient growth in the dairy environment, including lactose utilisation, and an extracellular protease required for milk-protein proteolysis as well as peptide transporters and proteins involved in stress resistance (Gorecki *et al*. 2011; Tarazanova *et al*. 2016). Recent comparative analyses of plasmid encoded functions in a larger panel of *L. lactis* strains further confirmed the richness of genes with biotechnological importance in the lactococcal plasmid complement (Ainsworth *et al*. 2014, Kelleher *et al*. 2019). A striking example is the examination of 12 plasmids in *L. lactis* FM03P, which revealed that no less than 10 of these plasmids encode genes that are predicted to be important for growth and survival in the dairy environment (van Mastrigt *et al*. 2018c). The plasmid encoded *L. lactis* repertoire includes not only a variety of metabolic functions (e.g. lactose utilisation, proteolysis and peptide import), but also genes involved in conjugation, bacteriocin production, exopolysaccharide synthesis and phage resistance (Kelleher *et al*. 2019). Intriguingly, the latter category not only included a variety of abortive infection functions (Ainsworth, Mahony and van Sinderen 2014; Kelleher *et al*. 2019), but also a range of genes encoding restriction modification systems (van Mastrigt *et al*. 2018c), as well as the first CRISPR-Cas system for adaptive phage resistance described for this species (Millen *et al*. 2012, 2019). Moreover, recent studies have identified plasmid encoded sortase C dependent pili, forming proteinaceous appendages on the lactococcal cell surface. Pilin formation in lactococci was previously associated with clinical- and plant-derived isolates (Oxaran *et al*. 2012). Engineered expression of pili in *L. lactis* strongly affected the cell's adhesion properties to different surfaces as well as human intestinal epithelial cells (Meyrand *et al*. 2013; Castelain *et al*. 2016). Although the latter observation does not reflect a normal habitat for *L. lactis*, this finding may indicate a role of the pili in adhesion to niche-specific surfaces, suggesting that lifestyle and niche adaptation may involve adjustments of pilin expression. Intriguingly, expression of pili drastically altered cell-surface hydrophobicity (Tarazanova *et al*. 2018) and influenced aggregation properties, which was reflected in the ~15-fold increased efficiency of conjugation of mobile genetic elements between pilin expressing strains (Tarazanova *et al*. 2016). Moreover, milk fermentation by such strains was shown to lead to increased viscosity and gel firmness of the fermented product and also increased the retention of *L. lactis* cells in curd rather than whey during the cheese making process (Tarazanova *et al*. 2018), which may both reflect the enhanced aggregation and/or milk–matrix interaction displayed by these pilin-expressing cells.

Taken together these findings underpin the importance of plasmid complement encoded functions in the dairy applications of the lactococci. Importantly, a recent analysis of the 'pan-plasmidome' of *L. lactis* revealed that at present this field has not reached saturation, implying that there are many plasmid-encoded functions that remain to be discovered in this species (Ainsworth *et al*. 2014; Kelleher *et al*. 2019). Analogously, a recent comparative genomics analysis of another class of mobile genetic elements in *L. lactis*, the integrative conjugative elements (ICEs), revealed that lactococcal ICEs display a high degree of genetic plasticity and can be classified in seven ICE families (van der Els *et al*. 2020b). Similar to the lactococcal plasmids, the ICEs detected in *L. lactis* encode a variety of industrially relevant functions, including phage abortive infection, antimicrobial production and carbohydrate utilisation (van der Els *et al*. 2020a), further supporting the biotechnological importance of functions encoded by mobile genetic elements in the dairy lactococci.

A third model strain that has been used as a representative of plant-derived *L. lactis* spp. *lactis* is strain KF147, of which the genome analysis clearly reflected its adaptation to the plant environment. Among these adaptations, the most prominent is the presence of a large panel of genes encoding glycoside-utilisation pathways in this plant isolate compared with the dairy-derived strains (Siezen *et al*. 2008; Siezen *et al*. 2010). Growth of KF147 on plant-derived medium (*Arabidopsis thaliana* leaf tissue lysate) was shown to involve the expression of a large repertoire of these pathways, including the utilisation of xylose, arabinose, cellobiose and hemicellulose, reflecting the availability of these nutrient sources in habitats associated with plants (Golomb and Marco 2015). Additionally, this strain encodes a mega-enzyme system composed of nonribosomal peptide synthetases and polyketide synthases (hybrid NRPS-PKS) predicted to produce a hexapeptide-malonyl hybrid molecule (Leu-DLeu-Asp-DAsn-Gly-Malonyl$_3$-DAsp; Khayatt *et al*. 2020) that contributes to oxidative stress tolerance, and could enhance growth under oxidative conditions in plant-associated environments (Golomb *et al*. 2018).

THE FOUNDATION OF ACIDIFICATION, CARBON METABOLISM IN *L. LACTIS*

Fuelled by its important role in food preservation by fermentation, carbon metabolism and lactic acid formation in *L. lactis* has been a prominent subject of research. Contrary to the plant isolates, most dairy isolates of *L. lactis* display a relatively limited capacity to use different carbohydrates as substrates for growth, including glucose, mannose, fructose, galactose, lactose, maltose and cellobiose (Siezen *et al*. 2008; Wels *et al*. 2019). This has been proposed to reflect genome-decay associated with prolonged cultivation in nutrient-rich niches (Wels *et al*. 2019), such as milk, which has also been detected in the genome analyses of several other LAB that serve as workhorses in the dairy fermentation industry like *Streptococcus thermophilus* and *Lactobacillus helveticus* (Bolotin *et al*. 2004b, Hols *et al*. 2005; Callanan *et al*. 2008). Expression and activity of carbohydrate import systems in *L. lactis* is subject to catabolite control exerted by carbon catabolite protein A (CcpA) and HPr, which enables preferential utilisation of sugars in environments that contain more than one sugar (Fig. 1). This leads to diauxic growth that includes a lag phase during which cells adapt to the utilisation of alternative sugars once the preferred sugar is consumed. Intriguingly, the analysis of diauxic growth of *L. lactis* in media containing glucose and cellobiose revealed that only a fraction of the culture proceeds to utilise cellobiose once the glucose is depleted, illustrating that diauxic shifts may include aspects of culture heterogeneity and bet-hedging (Solopova *et al*. 2014).

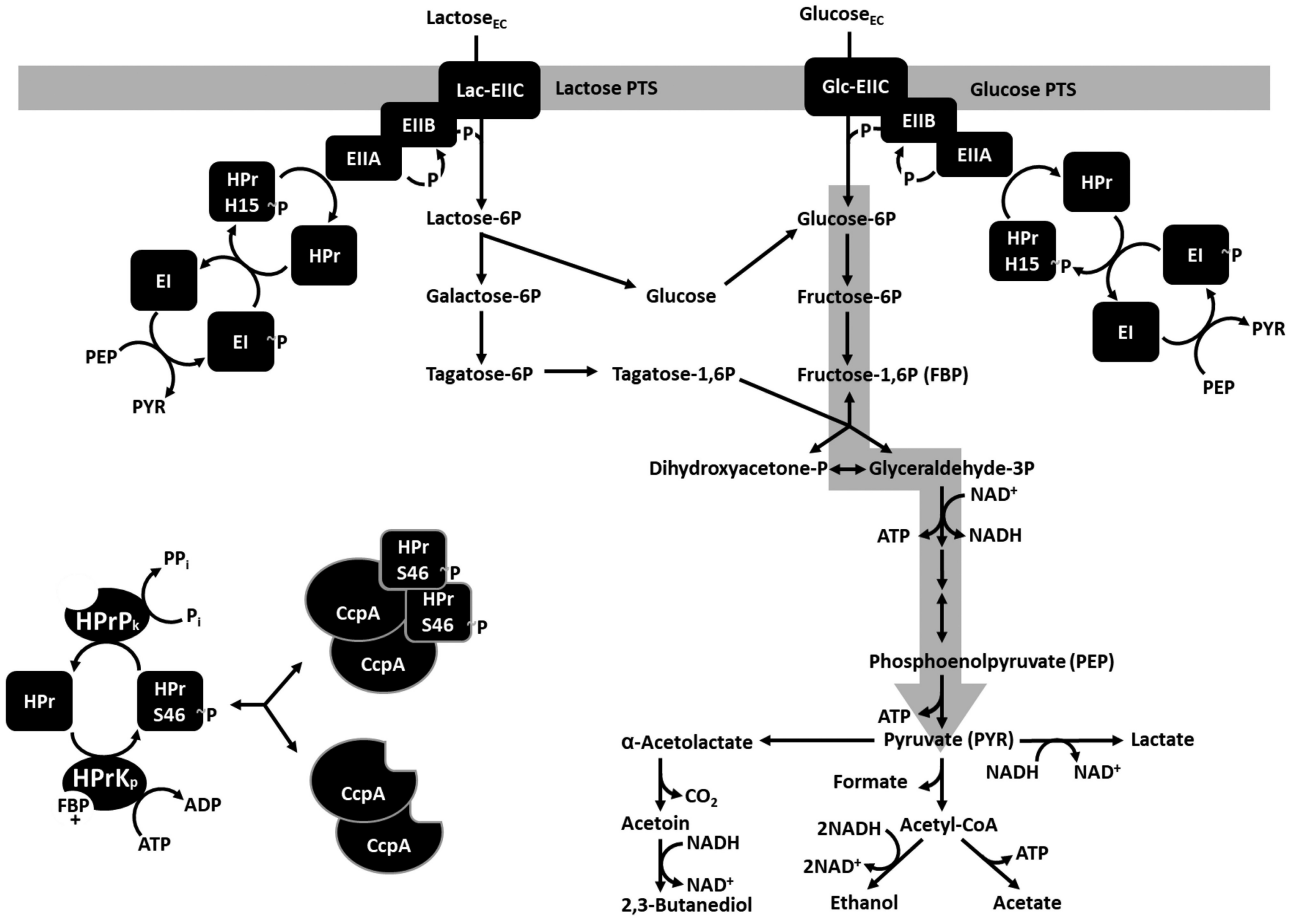

Figure 1. Schematic representation of the glucose and lactose utilisation pathway in *L. lactis*, including the differential pyruvate dissipation pathways known in this species. The scheme highlights the dualistic role of HPr and its differential phosphorylation in facilitating transport (HPr-HP) and catabolite regulation (HPr-SP) in concert with CcpA. The glycolytic pathway is highlighted with a grey background.

HPr plays a dualistic role in control of carbohydrate metabolism (for a review, see Deutscher, Francke and Postma 2006). On the one hand, it participates in the canonical EnzymeI-HPr phosphorelay that couples the glycolytic intermediate phosphoenolpyruvate (PEP) via phosphorylation on histidine residue 15 of HPr (HPr-HP) to substrate-specific phosphotransferase systems (PTS) that concurrently phosphorylate and import sugar. On the other hand, HPr can be phosphorylated on serine residue 46 (HPr-SP) by HPr kinase. Under conditions that support high glycolytic flux, the intracellular levels of the glycolytic intermediate fructose 1,6-bisphosphate (FBP) and ATP are increased, resulting in activation of HPr kinase activity leading to increased levels of HPr-SP. HPr-SP forms a complex with CcpA that plays a pleiotropic role in genome-wide regulation of carbohydrate utilisation (for reviews, see Titgemeyer and Hillen 2002; Warner and Lolkema 2003).

In addition to global carbon catabolite control, carbohydrate utilisation systems are additionally controlled by dedicated regulator proteins that often act as repressors, whose repressor function is eliminated when they bind a specific intermediate of the utilisation pathway of that carbohydrate (for a review, see Kok *et al.* 2017). Since lactose is the main carbon source encountered by *L. lactis* when growing in milk, its utilisation pathway has been studied in detail (Fig. 1). As indicated above, in many lactococcal strains lactose utilisation genes are plasmid encoded and a well-characterised example is present on the lactose mini-plasmid pMG820. The *lac*-operon on this plasmid encodes a lactose PTS import system (*lacEF*), a phospho-β-galactosidase (*lacG*) and the tagatose pathway (*lacABCD*) (de Vos *et al.* 1990; van Rooijen, van Schalkwijk and de Vos 1991), and its expression is controlled by the divergently transcribed repressor LacR, which relieves repression upon binding to the tagatose pathway intermediate tagatose-6-phosphate (van Rooijen, Gasson and de Vos 1992).

Once internalised, in *L. lactis* carbohydrates are processed through the Embden–Meyerhof–Parnas glycolytic pathway with different carbohydrates entering at different points in the pathway (Fig. 1). The enzymes involved in the pathway are generally highly expressed in *L. lactis*, which is in agreement with the genomic localisation of the encoding genes in close proximity of the genome origin of replication (Cocaign-Bousquet *et al.* 2002). In most growth conditions the glycolytic enzymes constitute a large proportion (reported to be up to 20%) of the overall cytoplasmic proteome (Puri 2014a; Goel *et al.* 2015). Expression of several glycolytic enzymes in *L. lactis* is regulated by the CcpA-HPr-SP complex, which upregulates the expression of phosphofructokinase (PFK) and pyruvate kinase (PYK) (Luesink *et al.* 1998; Zomer *et al.* 2007; Kok *et al.* 2017), as well as phosphoglucose isomerase (PGI), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase (Guedon, Jamet and Renault 2002). The control of glycolytic flux has been extensively studied and various control scenarios have been proposed that integrate gene

expression regulation with allosteric regulation of glycolytic enzymes by glycolytic intermediates as well as by intracellular levels or ratios of ATP/ADP + Pi and NADH/NAD^+. Overall, it has become clear that the lactococcal glycolysis runs at a very high (maximal) rate during balanced growth in batch cultures and remains highly active also in non-growing cells, demonstrating the uncoupling of central metabolism and growth (for reviews, see Koebmann *et al.* 2002; Liu *et al.* 2019). The glycolytic pathway leads to the formation of pyruvate, which under most conditions is almost exclusively dissipated to lactic acid involving lactate dehydrogenase (LDH), resulting in homolactic fermentation. However, pyruvate dissipation can also lead to the formation of other metabolic end-products like acetic acid, formic acid, ethanol (i.e. mixed acid fermentation), acetoin and butanediol (Fig. 1). These alternative metabolic end-products have been reported for specific growth conditions, including carbon limitation (Thomas, Ellwood and Longyear 1979), diminished rates of sugar catabolism (Cocaign-Bousquet *et al.* 1996) and aerobic conditions (Lopez de Felipe *et al.* 1998).

The linkage between glycolysis and pyruvate dissipation to lactic acid is reflected by the genetic linkage of the LDH encoding gene with the genes encoding the glycolytic enzymes PFK and PYK in the so-called *las* operon. Although the *ldh* gene is considered to be part of the *las* operon (i.e. the last of the three genes), a monocistronic *ldh* transcript has also been detected, which was initially proposed to derive from post-transcriptional processing of the polycistronic *las* operon transcript (Luesink *et al.* 1998), but was later proposed to depend on a separate promoter sequence upstream of the *ldh* gene (Bachmann *et al.* 2010). As described above, the *las* operon is subject to carbon catabolite regulation (Luesink *et al.* 1998; Zomer *et al.* 2007), aligning the expression of LDH and two key enzymes in the glycolytic pathway. In parallel, carbon catabolite regulation represses the expression of genes that encode functions involved in mixed acid fermentation like acetate kinase and pyruvate dehydrogenase (Zomer *et al.* 2007; Lopez de Felipe and Gaudu 2009). The consequences of CcpA-HPr-SP mediated regulation of pyruvate dissipation were underpinned by the observed reduced growth rate and mixed acid fermentation profile in a CcpA-deficient mutant of *L. lactis* MG1363 (Luesink *et al.* 1998). Thereby, carbon catabolite regulation plays a pleiotropic role in controlling carbon metabolism and fermentation in *L. lactis* that exceeds its canonical role in carbohydrate import regulation described in various bacteria (for reviews, see Titgemeyer and Hillen 2002; Warner and Lolkema 2003).

PYRUVATE DISSIPATION CONTROL

The main difference between homolactic and mixed acid fermentation lies in the energy yield, where pyruvate dissipation towards acetate yields an extra ATP in addition to the two ATP gained by the glycolytic pathway. It is quite striking that under anaerobic conditions and at higher growth rates *L. lactis* mainly displays 'energy-inefficient' lactic acid fermentation, despite the high energy demand of fast growth (Thomas, Ellwood and Longyear 1979; Flahaut *et al.* 2013; Goel *et al.* 2015). One would expect that at high growth rates lactococcal cells would employ their most energy-efficient metabolic pathway, which is also predicted by stoichiometry-based genome-scale metabolic models of *L. lactis* that predict carbon-flux distribution using highest energy and biomass yield as an objective (Teusink *et al.* 2009; Flahaut *et al.* 2013). These observations raise questions about the mechanism underlying the switching between homolactic and mixed acid fermentation in *L. lactis*, and imply that these bacteria have not evolved to optimised energy and biomass yield but rather towards maximised acidification rates (Teusink *et al.* 2009).

The intracellular redox balance, reflected by the NADH/NAD^+ ratio, plays an important role in controlling pyruvate dissipation in *L. lactis* (Garrigues *et al.* 1997; Lopez de Felipe *et al.* 1998; Lopez de Felipe and Gaudu 2009). The LDH-catalysed reduction of pyruvate to lactic acid regenerates NAD^+ from the glycolytically formed NADH, thereby, maintaining redox balance and sustaining high carbon flux and growth rate (Fig. 1). Indeed, LDH deficient mutants of *L. lactis* grow ~4- to 5-fold slower but reach a higher final cell density in batch cultures as compared with their parental strain under anaerobic conditions, where pyruvate is dissipated predominantly to acetoin/2,3-butanediol, ethanol and acetic acid (Platteeuw *et al.* 1995; Bongers *et al.* 2003). Moreover, LDH-deficient mutants also accumulate mannitol, which is formed from the glycolytic intermediate fructose-6-phosphate, involving mannitol-1-phosphate dehydrogenase that contributes to NAD^+ regeneration in these cells (Neves *et al.* 2000). However, when grown under aerobic conditions the LDH-deficient strain grows at almost the same rate as the wild-type strain, which is dependent on the activity of the endogenous water-forming NADH oxidase that regenerates NAD^+ and restores redox balance, utilising molecular oxygen as electron acceptor. In the presence of oxygen, the LDH-deficient strain produces predominantly acetic acid and acetoin, while ethanol formation and mannitol accumulation are to a large extent eliminated (Neves *et al.* 2000; Bongers *et al.* 2003). These results support that alternative NADH-utilising pathways are crucial in maintenance of redox balance in strains where lactic acid formation is compromised. Ethanol formation by alcohol dehydrogenase (ADH) plays such a role (Fig. 1), which was supported by the improved glucose fermentation observed in LDH-deficient lactococcal cells as a consequence of acetate co-metabolisation and its conversion to ethanol, with the concomitant NAD^+ regeneration (Hols *et al.* 1999b). Moreover, so-called 'redox engineering' by overexpression of NADH oxidase in *L. lactis* was shown to be an effective strategy to metabolically engineer wild-type (i.e. containing LDH) *L. lactis* towards almost exclusive production of acetic acid and acetoin (Lopez de Felipe *et al.* 1998). Employing the same strategy in an α-acetolactate decarboxylase deficient strain led to increased production levels of the butter aroma component diacetyl (Neves *et al.* 2000), whereas co-expression with pyruvate decarboxylase (derived from *Zymomonas mobilis*) increased the production levels of the yoghurt aroma component acetaldehyde (Bongers, Hoefnagel and Kleerebezem 2005). Collectively, these findings underpin the importance of the redox-balance reflected by the NADH/NAD^+ ratio in controlling lactococcal pyruvate metabolism.

The importance of LDH in the lactococcal lifestyle is further supported by the redundancy of genes encoding this function (Bongers *et al.* 2003; Gaspar *et al.* 2011). This is especially remarkable in the light of the small genome size of the lactococci and the fact that it has 3–4 LDH encoding genes. However, only the *ldh* gene encoded within the *las* operon is expressed at a high level, although it was reported that two of the alternative *ldh* genes (*ldhB* and *ldhX*) were also expressed at a low level (Gaspar *et al.* 2011). However, the expression of *ldhB* in *L. lactis* MG1363 was shown to become activated by promoter-up mutations upon prolonged anaerobic growth of a strain mutated in the *las* operon encoded *ldh*. In many cases, this expression activation depended on IS*981* mobilisation and its directional and precisely spaced insertion upstream of the *ldhB* gene, providing this gene with a restored promoter that depends on a IS*981*-derived −35 promoter region (Bongers *et al.* 2003). Regained LDH activity by *ldhB*

expression recovers lactic acid production that under anaerobic conditions coincides with the regain of growth rates comparable to the wild-type strain and loss of the increased biomass yield (Bongers *et al.* 2003). Engineered mutant strains of *L. lactis* MG1363 that lack the *ldh* gene of the *las* operon, or also the redundant *ldhB* and *ldhX* genes, have been extensively used for metabolic engineering to achieve highly efficient cell-factories for the production of various reduced end-products like alanine, mannitol and various 2,3-butanediol isomers (Hols *et al.* 1999a; Gaspar *et al.* 2011; Kandasamy *et al.* 2016; Liu *et al.* 2016, 2017, 2019). Taken together, these findings highlight the critical role of LDH in sustaining efficient and fast growth in *L. lactis* under anaerobic conditions. Finally, this notion is further supported by a study that employed a water-in-oil emulsion microdroplet technology to experimentally evolve *L. lactis* MG1363 towards maximised cell- and biomass yield rather than the maximised-growth and -acidification rate lifestyle of the wild-type strain. The evolved strains grew slower and reached higher culture densities driven by mutations that led to reduced rates of glucose import and glycolytic flux, which elicited a switch from homolactic to mixed acid fermentation leading to increased metabolic efficiency and biomass yield (Bachmann *et al.* 2013; Bachmann *et al.* 2017). In addition, a recent study revealed that these biomass-yield optimised strains also had an activated arginine utilisation pathway, further enhancing their metabolic capacity for energy generation to support biomass formation (Nugroho, Kleerebezem and Bachmann 2020). Notably, the reduced growth rate, increased biomass yield and acetic acid production resemble the physiological characteristics of *ldh* mutants of *L. lactis*, further supporting the pivotal role of lactic acid production by LDH in maintenance of the redox balance required to sustain high glycolytic flux and maximal growth (and acidification) rate.

The above indicates that the lifestyle of *L. lactis* is apparently optimised for fast growth and lactic acid production, a combination that is likely providing a competitive advantage in different environmental niches by the suppression of growth of competing microbes by rapid lactic acid accumulation and pH lowering. This characteristic is of fundamental importance for the fermentation industry, where fast preservation and shelf-life extension of the food raw-materials by acidification is one of the primary objectives.

DO PROTEOME CONSTRAINTS PLAY A ROLE IN ENVIRONMENTAL ADAPTATION?

To maintain optimal environmental fitness, bacteria need to adapt to changing conditions by changing their proteome composition. Proteome changes are required because overall protein synthesis and maintenance capacity of a cell is limited by cellular resources, disallowing the expression of optimal levels of all proteins at the same time. Such constraints can lead to fitness trade-offs, where optimisation of fitness-enhancing traits under one specific condition comes at the expense of fitness-enhancing traits under another condition. This results in so-called Pareto fronts that cannot be crossed (for a review, see Emmerich and Deutz 2018) forcing cells to regulate the adaptation of their proteome.

Bacterial proteome changes are predominantly driven by the adjustment of gene expression patterns, which is governed by regulatory networks involving single or multiple regulator proteins that respond to environmental cues. These regulatory networks have been extensively studied in *L. lactis*, in particular in model strain MG1363, and large amounts of gene regulation studies have contributed to the deciphering of the lactococcal 'genome-wide' gene regulation network (de Jong *et al.* 2013; Kok *et al.* 2017; Omony *et al.* 2019). In addition, several studies involving proteome analyses have examined and modelled the determinants of protein levels, translation efficiency and metabolic costs associated with protein synthesis and maintenance (Dressaire *et al.* 2009; Dressaire *et al.* 2010; Lahtvee *et al.* 2011; Lahtvee *et al.* 2014).

Basic models of self-replicating systems indicated that a trade-off between enzyme costs and metabolic benefits, i.e. ATP yield, could explain the apparent contradiction of energetically inefficient homolactic fermentation observed in fast-growing and thereby high-energy-demanding *L. lactis* (Molenaar *et al.* 2009). Importantly, the model took the costs related to the synthesis of enzymes required for a specific metabolic pathway into account. In contrast, traditional approaches such as Flux Balance Analysis of genome-scale metabolic models only use reaction stoichiometry, thereby disregarding the costs of production of the enzyme involved in the underlying metabolic pathways. Although the model was constructed to generically represent self-replicating systems, its extrapolation to *L. lactis* predicted that the 'extra' investment involved in biosynthesis of the acetic acid formation pathway is unfavourable at high growth rates (Molenaar *et al.* 2009). Notably, similar proteome efficiency effects that can be expressed as the amount of ATP produced per amount of protein, were later shown to explain overflow metabolism in *Escherichia coli*. (Basan *et al.* 2015). However, proteome investigation of *L. lactis* MG1363 at different growth rates established that the enzymes involved in acetic acid production were present at equal levels in homolactic fast-growing cells and slow-growing cells that displayed a mixed acid fermentation profile, which appeared to falsify the protein cost-benefit prediction by the model (Goel *et al.* 2015). It is important to note that the proteome study employed glucose-limited chemostat cultivation conditions that are expected to have led to relief of carbon catabolite repression, which may have influenced the levels of expression of the acetic acid and ethanol formation pathways (see above). Nevertheless, the obtained results establish that, although present, the acetic acid formation pathway remains unemployed by *L. lactis* during fast growth, suggesting that growth rate is not strictly constrained by proteome optimisation under these conditions.

Do these findings indicate that constraints in resource allocation and proteome optimisation are not applicable as the predominant determinant of growth rate and/or fitness trade-offs in *L. lactis*? The most likely answer to this question is 'no'. A possible explanation could be that primary constraint is not the cytoplasmic proteome of *L. lactis*, but rather the membrane proteome and/or the cellular transport capacities. This notion appears to be supported by several experimental evolution studies performed with *L. lactis* MG1363 (Smith *et al.* 2012; Bachmann *et al.* 2013; Price *et al.* 2019). An interesting example is the selection of *L. lactis* mutants with an improved heat-stress resistance phenotype, which coincided with the appearance of a salt-hypersensitivity phenotype (Smith *et al.* 2012). The evolved strain carried a mutation in the gene encoding GdpP, which is a membrane bound protein involved in stress signalling via its cyclic dimeric AMP (c-di-AMP)-phosphodiesterase activity that modulates the cytoplasmic levels of c-di-AMP (Smith *et al.* 2012). The c-di-AMP molecule serves as a second messenger that plays a critical role in regulation of a variety of phenotypes, including osmotic-stress tolerance, in various bacteria (Yin *et al.* 2020). In *L. lactis* c-di-AMP was shown to affect osmotic homeostasis by controlling transporters associated with potassium

homeostasis and import of osmoprotectants (Pham *et al.* 2018; Pham and Turner 2019) as well as by its connection to peptidoglycan biosynthesis (Zhu *et al.* 2016). The mechanism underlying the effect of GdpP-mutation on heat-stress tolerance remains unclear, but it is intriguing that in *Streptococcus pneumoniae* mutations in two heat shock suppressors were shown to induce changes of c-di-AMP levels and undermine the toxicity associated with this second messenger (Zarrella, Metzger and Bai 2018). The finding that both stress tolerance systems are connected to c-di-AMP in *L. lactis*, combined with the prominent role of this compound in controlling membrane proteome function, would support the hypothesis that membrane proteome constraints underlie the observed heat- and salt-stress fitness trade-off.

In addition, experimental evolution to obtain *L. lactis* MG1363 mutants that were either optimised for biomass yield rather than growth rate (Bachmann *et al.* 2013) or displaying improved fitness under glucose limitation (Price *et al.* 2019) led to the isolation of mutants with an altered membrane proteome that resulted in opposing adjustments of glucose import capacity. The biomass-yield optimised strains that were selected using a water-in-oil droplet culturing regime displayed a typical mixed acid fermentation that coincided with an ∼25–40% reduced acidification rate. The study identified an amino-acid change in a component of the only high-affinity glucose PTS (Castro *et al.* 2009), PtnD (F65L), that led to reduced glucose import rates observed in this strain (Bachmann *et al.* 2013). These findings agree with the hypothesised membrane proteome constraint underlying the investigated trade-off between growth rate and biomass yield. Mutation analysis of the evolved strains selected for improved fitness under glucose limitation, identified amino acid substitution in CcpA (M19I or M19T) as the causative mutation. The mutation altered the affinity of CcpA for specific sequence-variants of its target *cis*-acting element, the *cre*-box. The CcpA affinity change led to a modified pattern of expression, including both increased and decreased transcriptional activity of multiple genes belonging to the CcpA regulon, particularly affecting the transcriptional landscape of genes encoding carbohydrate transport systems. The transcriptional changes included the elevated expression of the *ptn* operon encoded high-affinity glucose PTS, in parallel with reduced expression of the *ptc* operon encoded low-affinity glucose PTS (Castro *et al.* 2009). These changes in glucose import system expression led to a 3-fold accelerated glucose import rate in the evolved strain, which explained the improved fitness under glucose-limiting conditions. The pleiotropic effects on the CcpA-regulon were also shown to negatively affect the capacity of the evolved strain to utilise alternative carbon sources due to reduced expression levels of the import systems for these sugars (Price *et al.* 2019). Thereby this evolved strain has become more specialised for conditions with limited glucose availability, but at the same time has apparently lost part of its adaptability to environmental conditions that contain alternative carbon sources.

These experimental evolution studies agree with the hypothesis that constraints in resource allocation, proteome composition and transport functions are associated with boundaries in the membrane proteome of *L. lactis*.

ADAPTATION TO THE DAIRY NICHE

The adaptation of *L. lactis* to the dairy niche has been studied in many ways, each of them addressing specific aspects of the *L. lactis* lifestyle during its most prevalent biotechnological application.

The comparative analysis of *L. lactis* genomes confirms the hypothesis that dairy isolates have evolved from plant isolates, which is exemplified by the dairy isolates are characterised by the loss of several glycoside utilisation gene clusters that are dispensable in the dairy niche but are important for growth on plant material (Siezen *et al.* 2008; Passerini *et al.* 2010; Bachmann *et al.* 2012; Price *et al.* 2012; Cavanagh, Fitzgerald and McAuliffe 2015; Golomb and Marco 2015; Wels *et al.* 2019). In general, the genomic adaptations to the dairy environment appear to be predominated by events of loss of function, which is contrasted by the observation that gain of function in dairy adapted *L. lactis* strains is often associated with the acquisition of mobile genetic elements, i.e. plasmids (see also above). As an example of dairy-associated loss-of function, it was recently established that engineered expression of the regulator ComX could activate the competence phenotype in specific *L. lactis* plant isolates (David *et al.* 2017; Mulder *et al.* 2017), whereas a variety of disruptive mutations in late-competence genes was found in all *L. lactis* strains isolated from the dairy environment (Wydau *et al.* 2006; Mulder *et al.* 2017). This finding suggests that natural competence could compromise environmental fitness of *L. lactis* in the dairy environment, which is a striking contrast with the consistent presence and functionality of natural competence associated genes in another dairy LAB-workhorse, *Streptococcus thermophilus* (Fontaine *et al.* 2010; Fontaine *et al.* 2013). The loss of competence in dairy adapted *L. lactis* strains is possibly related to the activation of competence expression under carbon-starvation conditions (Redon, Loubiere and Cocaign-Bousquet 2005; Ercan *et al.* 2015a) that may occur during cheese production (see also below). This suggestion is supported by the reported activation of *comX* expression during cheese production (Bachmann *et al.* 2010). Although the precise environmental trigger and regulatory mechanism involved in competence activation in *L. lactis* remain to be deciphered, competence activation during dairy fermentation could lead to genomic instability, which could provide the selective pressure that favours the loss of this function.

The genome adaptations of plant-derived *L. lactis* isolates to the dairy environment (e.g. a process termed 'domestication') were investigated by experimental evolution that involved 1000 generations of growth of the plant isolate *L. lactis* subspecies *lactis* KF147 in milk. The original strain (Siezen *et al.* 2010) does not grow efficiently in milk but prolonged cultivation in this medium allowed the isolation of mutant derivatives that displayed a significantly improved growth rate in milk (Bachmann *et al.* 2012). The genome analysis of these 'domesticated' mutants revealed gain of function mutations in amino acid biosynthesis and transport functions as well as the loss of an integrated conjugative element (ICE) that encodes functions associated with growth on plant derived materials (Bachmann *et al.* 2012). Moreover, comparative transcriptome analyses of the original KF147 strain, its milk-adapted derivatives and a typical dairy isolate of *L. lactis* confirmed that the 'domesticated' mutants displayed an increased transcriptome-resemblance with the dairy isolate. Relative to its parental strain KF147, the transcriptional changes observed in the milk-adapted mutants reflected its adaptation to the dairy niche, including the activation of various nitrogen metabolism functions and the repression of many carbohydrate utilisation pathways (Bachmann *et al.* 2012). These genomic adaptation signatures clearly illustrate that experimental domestication approaches can recapitulate some of the genome differences detected by comparative genomics of *L. lactis* strains isolated from different environmental niches. However, it should be noted that this experimental evolution experiment 'only' encompassed 1000 generations of growth in

milk, while the hypothesised progressive loss of glycoside utilisation genes is likely to require prolonged cultivation in milk.

ADAPTIVE RESPONSES DURING DAIRY APPLICATIONS

The transcriptional responses of *L. lactis* during growth in milk and cheese production conditions have been investigated. *Lactococcus lactis* is especially important in the production of semi-hard cheeses, where it plays a key role in texture and flavour properties of the fermented product (see above). The environmental conditions encountered during cheese production and ripening are highly dynamic and depend on the specific protocol employed for production of different semi-hard cheeses like Cheddar or Gouda. These protocols expose the lactococci to variable temperature regimes, rapid decreases in pH by conversion of lactose to lactate, followed by carbon starvation during ripening, as well as increases in osmolarity during brining, and decreases in water activity after curd separation from whey (Fig. 2).

Various studies focused on the response and coping mechanisms of *L. lactis* using a variety of stress conditions that are considered important during cheese production processes. This has led to a relatively comprehensive view of overall stress responses in this species (for a review, see Papadimitriou *et al.* 2016). However, most stress-response studies addressed a single environmental stress and often employed laboratory media rather than milk. Although these studies may adequately reflect part(s) of the lactococcal response to dairy applications, they fail to recapitulate the application's environmental complexity and its impact on global gene expression control. Several studies have described the responses of *L. lactis* to growth and acidification in milk using transcriptome (Raynaud *et al.* 2005; de Jong *et al.* 2013; Larsen, Brosted Werner and Jespersen 2016a, Larsen *et al.* 2016b) and proteome (Gitton *et al.* 2005) analyses. These studies highlighted dynamic changes in expression of carbohydrate-, nitrogen- and nucleotide-metabolism as well as ion-transport and stress response functions, indicating the wide-ranging impact of growth in milk on the overall physiology of *L. lactis*. The observed responses included the anticipated increased expression of peptide transport systems, peptidolytic enzymes and amino-acid biosynthesis and interconversion functions, which were especially apparent at later timepoints of fermentation (Gitton *et al.* 2005; de Jong *et al.* 2013; Larsen *et al.* 2016b). In addition, several studies highlight the important transcriptional consequences of oxygen presence in milk and its depletion during early stages of lactococcal growth (de Jong *et al.* 2013; Cretenet *et al.* 2014; Larsen, Brosted Werner and Jespersen 2016a, Larsen *et al.* 2016b). However, the results of these studies are difficult to compare because they employ different milk-medium preparations, culturing regimes and sampling timepoints. Moreover, and maybe most importantly, these studies use different reference samples for the normalisation of the data obtained during growth in milk. Finally, only one study presents an integrative view of the transcriptome profiles during growth in milk and uses the data to reconstruct a time-resolved gene-regulation network (de Jong *et al.* 2013). In contrast, the other studies investigated transcriptional profiles at only a few timepoints during growth in milk, which provides important, but limited, insights in the dynamics of milk-adaptive behaviour of *L. lactis*.

To decipher the adaptive response of *L. lactis* during cheese production and ripening, an adapted recombinase-based *in vivo* expression technology (R-IVET) screening (for a review, see de Vos, Bron and Kleerebezem 2004) was implemented in model strain MG1363. The screening system combined the typical R-IVET antibiotic-resistance based selection of *in situ* activated promoters with the downstream quantitative *in situ* analysis of promoter activity in a time-resolved manner, employing the bacterial luciferase encoding *luxAB* genes as promoter probe (Bachmann, Kleerebezem and van Hylckama Vlieg 2008). This R-IVET approach was employed in different LAB to identify promoters that are activated during dairy application conditions (Bachmann *et al.* 2010; Junjua *et al.* 2014), including the time-resolved analysis of promoter activation in *L. lactis* MG1363 during Gouda-type cheese production conditions (Fig. 2) using a miniaturised, 96-well-based cheese production set-up (Bachmann *et al.* 2009, 2010). The study identified close to 100 promoter elements in the *L. lactis* MG1363 genome that were activated during cheese production and ripening compared with laboratory culture conditions. Analysis of the genes under control of these *in situ* activated promoters highlighted adaptation in carbon metabolism, in particular the activation of the pentose-phosphate pathway, as well as the activated expression of nucleotide import systems, indicative of nucleotide starvation during the application in cheese. The data also prominently supported the activation of amino acid and peptide transport as well as several amino acid biosynthesis and interconversion pathways (Bachmann *et al.* 2010), which were proposed to be controlled by the global nitrogen metabolism regulator CodY (for a review, see Kok *et al.* 2017) and further confirmed the importance of adaptation of nitrogen metabolism during cheese production. Moreover, brining activation was observed for promoters located upstream of genes involved in acid tolerance that have been reported to be inducible by chloride exposure (Sanders *et al.* 1998), confirming the accuracy of the R-IVET screening model to reflect the environmental adaptations of the gene expression repertoire in these cells. Even though *L. lactis* MG1363 lacks several functions, disallowing it to grow autonomously in milk, this study started to unravel the *in situ* behaviour of *L. lactis* in an unprecedented time-resolved manner, which provided a first glimpse at this species' adaptive behaviour during cheese production.

With the decreasing costs involved in high-throughput sequencing, recent studies have started to assess the population dynamics and *in situ* gene expression of complex cheese starters during cheese production, using metagenomic and metatranscriptomic approaches. Several studies reported on the LAB-population dynamics during industrial and artisanal cheese production, detecting not only the starter culture-derived bacteria but also the non-starter LAB that are particularly important in several cheese types. These studies have targeted a variety of different cheese products including the Dutch Gouda (Erkus *et al.* 2013; Erkus *et al.* 2016), and Maasdam (Swiss-type) cheeses (Duru *et al.* 2018; Mataragas 2020), as well as different (artisanal) cheeses from other geographic origins, including Italian (De Filippis *et al.* 2016), French (Dugat-Bony *et al.* 2015), Mexican (Escobar-Zepeda, Sanchez-Flores and Quirasco Baruch 2016) and Belgian (Delcenserie *et al.* 2014) cheeses. In several of these systems, (multiple) *L. lactis* strains are dominating the microbial community during cheese production and metagenome analysis offers insight in their abundance over time, while metatranscriptome analysis can assess their gene expression over time. Comparative metatranscriptome analysis to decipher the general lactococcal gene expression profiles during cheese production is complicated by the different strains used in these processes, as well as the variable production regimes of the different cheese-types. Moreover, analogous to

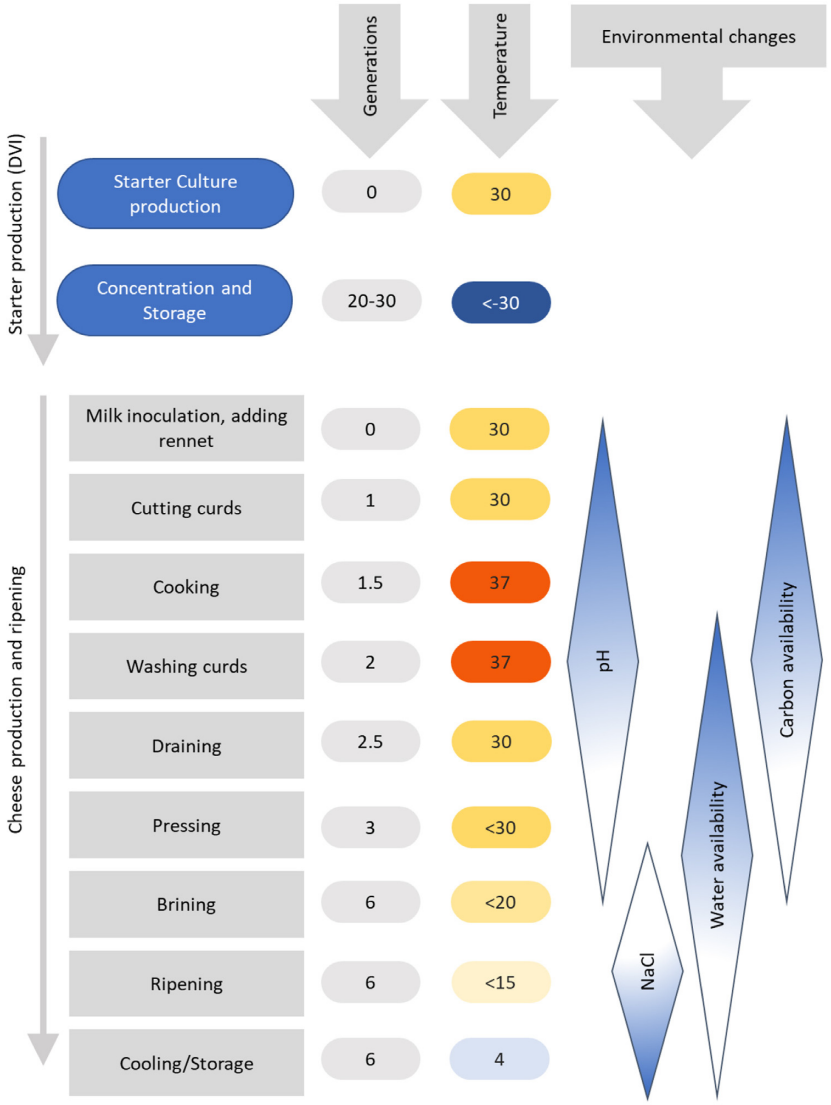

Figure 2. Schematic representation of starter culture production for direct vat inoculations and a Gouda-type cheese manufacturing protocol. The generations column is an estimate of the cumulative number to generations made by the starter culture during cheese making. Starter culture cells are exposed to rapid environmental changes such as temperature, pH, salt, water availability and sugar concentrations (periods of change are indicated).

the transcriptome studies performed for *L. lactis* grown in milk (see above), the number of timepoints that is generally analysed in these studies is quite limited and provides not more than a snapshot of the lactococcal activity profile. For example, the study of the lactococcal gene expression profile during Maasdam cheese production employed triplicate samples taken at two stages of the ripening period, i.e. after 12 days of the 30-day initial 'warm-room' (20°C) ripening and 7 days after the transfer to the second stage 30-day ripening period in the 'cold-room' (5°C) (Duru *et al.* 2018). Although the study does not allow true time-resolved expression analysis, it did reveal the sustained expression of central carbon metabolism associated genes in *L. lactis*, also during the cold-room ripening period. This sharply contrasted the observed decline of activity seen for the other members of the microbial community involved in the production of this type of cheese, which include *Lactobacillus rhamnosus*, *Lactobacillus helveticus* and *Propionibacterium freudenreichii* that collectively represent 10–20% of the overall cheese microbiota (Duru *et al.* 2018). This finding implies that the propionic acid fermentation that is of importance in the ripening of this type of cheese appears to be constrained to the ripening period at 20°C where the responsible organism (*Propionibacterium freudenreichii*) is still actively expressing its metabolic functions. Despite the limitations of the presently available studies, the pioneered metagenome and metatranscriptome approaches are appropriate to unravel the adaptive behaviour of starter cultures during the dynamic environmental conditions encountered during cheese production processes. It is important to align approaches and technologies applied in different cheese model systems, to enable the comparative analysis of lactococcal adaptation to different cheese environments and to eventually decipher the common and/or product-specific responses of the species to these dairy applications. Improved understanding of the activity profiles of *L. lactis* and other LAB during cheese ripening may offer new approaches to use ripening marker-genes for the selection of novel starter strains with improved ripening

performance, or could provide clues towards alterations in the production process conditions that modulate or accelerate ripening (De Filippis *et al.* 2016).

THE IMPORTANCE OF MICROBIAL DORMANCY IN CHEESE PRODUCTION

In natural reservoirs, *L. lactis* cells probably spend more time in a non-growing state than in intermittent periods of (fast) growth. Similarly, during most of the cheese ripening period, *L. lactis* is also in a senescent or dormant state, which is due to the low-level availability and rapid depletion of lactose during cheese production (Fig. 2; see also below). There is only limited knowledge of the physiology of lactococcal cells in such a dormant state. Most of our knowledge about *L. lactis* physiology is based on studies employing batch cultures grown in laboratory media that are rich in carbon sources and allow rapid acidification, and where the eventual arrest of growth is caused by the lowering of pH in combination with the accumulation of organic acid.

The dormancy state in bacterial cultures is characterised by two commonly emerging physiological states, e.g. the persister and the viable-but-not-culturable (VBNC) (sub)populations. VBNC (sub)populations retain metabolic activity but are unable to spontaneously resume growth when transferred to growth-supporting conditions, whereas persister (sub)populations are able to do so (for reviews, see Hayes and Low 2009; Oliver 2010; Ayrapetyan, Williams and Oliver 2018). Dormancy states have been described for many microorganisms and are considered the prevalent state in various natural environments, which is exemplified by the reported fraction of dormant cells in soil samples that can exceed 80% (Lennon and Jones 2011). Microbes have been described to enter this dormancy state upon the exposure to stressful conditions, such as an unfavourable temperature or pH, the presence of antibiotics or starvation (Ayrapetyan, Williams and Oliver 2018). The evolutionary relevance of dormancy states is probably related to the improved stress resistance of dormant cells, whereby fitness and survival is improved in environments that are variable and include transient and/or long-term harsh conditions that are incompatible with growth. This is underpinned by the observation that VBNC *L. lactis* cells are able to survive for long periods of time, i.e. for >10 years (Weimer 2011).

VBNC cells have been described to emerge in cultures of *L. lactis* and other LAB in response to carbon starvation and low pH (Ganesan, Stuart and Weimer 2007; El Arbi *et al.* 2011; van Mastrigt *et al.* 2018a) and during food production processes such as cheese ripening (Ruggirello, Dolci and Cocolin 2014). It has been demonstrated that carbon starved VBNC cells can still transcribe genes and utilise amino acids as carbon and energy source, which leads to the production of flavour compounds such as branched-chain fatty acids (Ganesan, Dobrowolski and Weimer 2006). In addition, the contribution of VBNC cells to bioconversion of raw materials has been demonstrated, where for example, it was shown that VBNC *Lactobacillus hilgardii* can contribute to malolactic fermentation occurring during apple cider production (Quirós *et al.* 2009).

Recently, it was described that exposure of *L. lactis* MG1363 to ampicillin leads to the emergence of both VBNC and persister subpopulations (van Tatenhove-Pel *et al.* 2019). Ampicillin-induced persister cells represented only a minor subpopulation (0.3%) when exponentially growing cultures were used, but increased substantially (7.6%) when cells were obtained from the stationary phase of growth (van Tatenhove-Pel *et al.* 2019). The simultaneous co-occurrence of VBNC and persister subpopulations has also been described for other organisms (Orman and Brynildsen 2013; Ayrapetyan *et al.* 2015) and mechanisms that have been proposed to explain this heterogeneous culture response include the involvement of toxin–antitoxin systems, ppGpp-mediated stringent responses and intracellular ATP levels. However, none of these mechanistic explanations adequately explains the dormancy continuum that includes VBNC and persister subpopulation formation in *L. lactis* (van Tatenhove-Pel *et al.* 2019).

Taken together, these findings indicate that it is important to better understand the physiology of dormant *L. lactis* cells that can emerge during cheese ripening and/or carbon starvation, or by near-zero growth rate conditions (van Mastrigt *et al.* 2018a). Therefore, the investigation of *L. lactis* cells that are in a dormant state or growing at extremely low rates could provide an appropriate impression of the lactococcal physiology during cheese production and ripening. Mimicking such conditions in the laboratory may serve as a proxy for the quantitative assessment of lactococcal metabolic activities under cheese ripening conditions, including its capacity for flavour formation.

ADAPTIVE RESPONSES TO ZERO GROWTH; RETENTOSTAT CULTIVATION

Retentostat cultivation provides an approach to study the adaptation of microorganisms to long periods of nutrient limitation and extremely low growth rates. A retentostat is a modification of the well-established chemostat cultivation method, in which a biomass filter is connected to the effluent line to retain the biomass inside the bioreactor. Upon prolonged retentostat cultivation, the culture reaches a stage where all nutrient-derived energy is allocated to maintenance and growth completely stagnates, which is referred to as zero growth. The physiological adaptation to zero growth has been investigated in several LAB, including different strains of *L. lactis*, but also *Leuconostoc mesenteroides* and *Lactobacillus plantarum* (Goffin *et al.* 2010; Ercan, Smid and Kleerebezem 2013; van Mastrigt *et al.* 2018a, 2019a). During long-term retentostat cultivation the growth rate of *L. lactis* decreased to 0.0006–0.001 h^{-1}, corresponding to generation times of a month to even a year. These cultures consistently retained a high level of viability according to live/dead staining analysis, although part of the population appeared to have entered a VBNC state (Ercan, Smid and Kleerebezem 2013; van Mastrigt *et al.* 2018a).

Remarkable differences in maintenance energy requirement were reported for the *L. lactis* plant isolate KF147 and the dairy isolate FM03-V1. The common approach to estimate maintenance energy requirements of a microbe is based on the extrapolation of the energy requirements in chemostat cultivations at different growth rates. For *L. lactis* KF147 this estimate was very similar to the maintenance energy determined by prolonged retentostat cultivation (Ercan, Smid and Kleerebezem 2013), whereas for *L. lactis* FM03-V1 this estimate was >6-fold higher than that determined when cells were in a zero growth state. This observation was further confirmed by growing *L. lactis* FM03-V1 at a rate below 0.025 h^{-1} using a partial cell-recycling chemostat (van Mastrigt *et al.* 2019b). Comparison of the maximum biomass yield and maintenance requirements of the plant and dairy *L. lactis* strains revealed that the plant-isolate KF147 has an ~2-fold reduced energy requirement for biomass formation and spends ~4-fold less energy on maintenance when cultured at growth rates higher than 0.1 h^{-1}. Nevertheless, the

maintenance energy requirement in a zero growth state was very similar for both strains (Ercan, Smid and Kleerebezem 2013; van Mastrigt *et al.* 2018a). Another difference observed during retentostat cultivation of the two *L. lactis* strains was their pyruvate dissipation profile, where *L. lactis* FM03-V1 consistently displayed the mixed acid fermentation profile that is expected during low growth rate and glucose limitation (van Mastrigt *et al.* 2018a), whereas *L. lactis* KF147 displayed an unstable pyruvate dissipation profile that fluctuated between homolactic and mixed acid fermentation over time (Ercan, Smid and Kleerebezem 2013), which remains to be understood.

These strain-specific energy requirements for growth and maintenance imply that the plant isolate *L. lactis* KF147 is continuously in an energy-saving mode, whereas the dairy isolate *L. lactis* FM03-V1 only enters such mode in a zero growth state. This physiological difference may reflect the long-term adaptation of the plant isolate to the energy-poor plant environment and/or the dairy isolate to the energy-rich dairy environment. The latter suggestion is supported by the observation that the energy household of another dairy-isolated LAB, *Leuconostoc mesenteroides* FM06, resembled that of *L. lactis* FM03-V1 (van Mastrigt *et al.* 2018a, 2019a).

PROTEOME ADAPTATION AND MANAGEMENT DURING ZERO GROWTH

It has been proposed that protein turnover is the main determinant of energy requirement in *L. lactis* (Lahtvee *et al.* 2014; Kempes *et al.* 2017). Based on studies of the behaviour of *L. lactis* during retentostat cultivation, it was hypothesised that energy-saving adaptations in these bacteria are driven by changing proteome management from the energetically costly breakdown and subsequent re-synthesis of damaged proteins, towards disaggregation of damaged proteins and their chaperone-assisted refolding (Mogk, Bukau and Kampinga 2018). This hypothesis is supported by time-resolved transcriptome studies of *L. lactis* KF147 during more than a month of retentostat cultivation (Ercan *et al.* 2015b), revealing the progressive upregulation of stress response functions. These functions included the chaperones ClpB, DnaK, DnaJ and GrpE that cooperate in disaggregation and refolding of proteins, as well as in the upregulation of the so-called hibernation-promoting factor YfiA (Puri *et al.* 2014b) that increases ribosome dimerisation and thereby lowers translation (Fig. 3). This reduced translation capacity is in agreement with the reduced expression during zero growth of components of the translational machinery, including ribosomal protein and amino acyl tRNA synthases. Notably, stress response activation and reduced translation capacity appears to be a conserved response to retentostat cultivation in various microbes, including *Bacillus subtilis, Saccharomyces cerevisiae* and *Aspergillus niger* (Jørgensen *et al.* 2010; Boender *et al.* 2011; Overkamp *et al.* 2015; Ercan *et al.* 2015c). Refolding of damaged proteins and slowing-down protein translation reduces the energy requirements associated with *de novo* protein biosynthesis and is beneficial during long periods of nutrient limitation. However, these adaptations have a trade-off because they reduce the adaptability of the proteome that is required to enable growth in new environments, which may (in part) underlie the observed inability of the VBNC population to form colonies when plated on rich media. However, this trade-off is (in part) compensated by the progressive relief of repression of several operons involved in carbohydrate import and utilisation that occurs during prolonged retentostat cultivation of *L. lactis* KF147, and which was shown to support the accelerated utilisation of these carbohydrates when they become available (Ercan *et al.* 2015a,b). This kind of lifestyle adaptation towards a more generalised phenotype has also been observed in other microorganisms during prolonged retentostat growth (Ercan *et al.* 2015c) and is a typical adjustment observed in microbes that live in environments that are notoriously poor in nutrients (Egli 2010; Hobbie and Hobbie 2013). In the context of energy-saving adaptations that occur in proteome management during zero growth conditions, it is quite remarkable that *L. lactis* FM03-V1 appears to maintain its plasmid complement with unaltered copy-numbers under these conditions, which underpins the autonomy of the replication of these extrachromosomal elements (van Mastrigt *et al.* 2018d).

The transcriptome analysis of *L. lactis* K147 during retentostat cultivation revealed that besides the elevated expression of the *dnaKJgrpE* operon also other stress responses were induced, including heat shock (*groELS*) membrane-associated stress (*hemG*, *lmrA*, *rmaB*, *yhbF*), acid (*uvrB*, *uspA*, *rcfB*), cold shock (*cspA1*, *cspB1*, *cspC1*, *cspD*, *cspA2*, *cspB2*, *cspC2*) and membrane-associated stress (*hemG*, *lmrA*, *rmaB*, *yhbF*) (Ercan *et al.* 2015b). Stress tolerance analysis indicated that these responses corresponded to >1000-fold higher resistance to heat and acid stress and an ~10-fold higher resistance to oxidative stress (Ercan 2014). Notably, the expression level of these stress-related genes displayed a strong inverse correlation with the growth rate of *L. lactis* KF147, exemplifying the trade-off between growth rate and stress robustness in *Lactococcus lactis* (Ercan 2014).

ZERO GROWTH CONDITIONS AS A REFLECTION OF CHEESE RIPENING PHYSIOLOGY

Cheese ripening involves long-term incubation periods that are required for the development of flavour volatile profiles that are determining the taste of the cheese. Ripening may take several years, e.g. up to 3 years for the production of Parmigiano-Reggiano cheese. Thereby, the contribution of starter cultures, including *L. lactis*, to the formation of these flavour volatiles is largely dependent on cells that are in a zero growth state. Importantly, the conversion of amino acids into cheese-relevant flavour volatiles was reported to be higher in non-growing compared with growing *L. lactis* cells (van de Bunt *et al.* 2014), and the amino-acid catabolic pathways involved were shown to be induced under starvation conditions that coincide with the emergence of VBNC (sub)populations (Ganesan, Stuart and Weimer 2007). Therefore, analysis of flavour formation capacities during zero growth could provide more insight in the flavour-forming capacities of *L. lactis* during cheese ripening.

Zero growth cultures of *L. lactis* FM03-V1 produced several aroma compounds that are also present in cheese produced with this strain, while fast-growing cells of this strain failed to do so. These flavour compounds included several amino acid catabolism-derived metabolites of which the production rate was progressively increasing during prolonged retentostat cultivation (van Mastrigt *et al.* 2018a,b). *Lactococcus lactis* KF147 displayed a progressively declining amino acid consumption level during the initial 3 weeks of retentostat cultivation, which is likely to reflect the decreased amino acid requirement for protein synthesis when growth rate declines towards near-zero growth rates (Ercan *et al.* 2015a,b). However, prolongment of retentostat cultivation led to elevated consumption of amino acids, which coincided with the appearance of VBNC subpopulations in the culture (Ercan, Smid and Kleerebezem 2013).

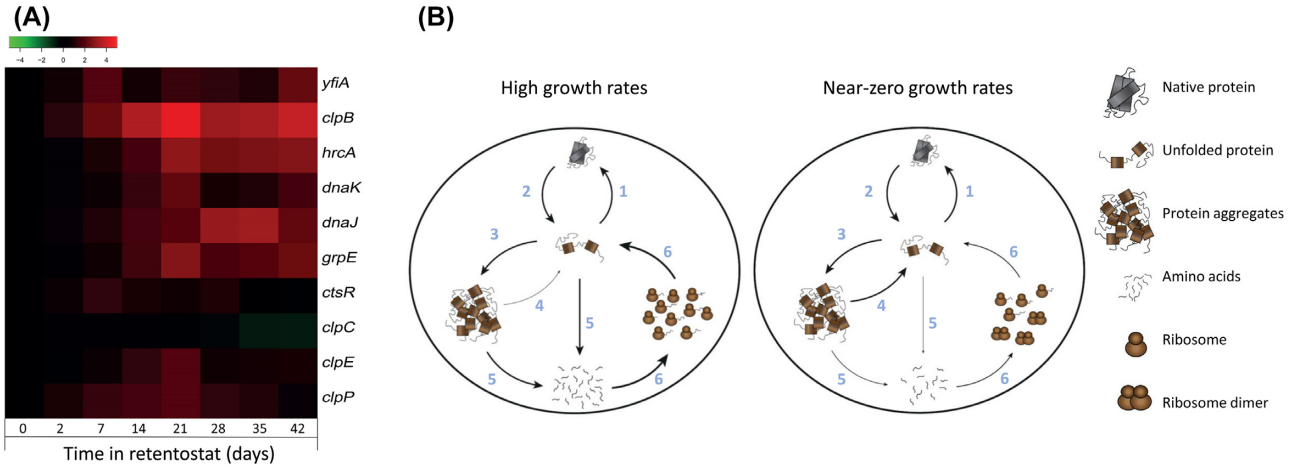

Figure 3. Proteome management at near-zero growth rates is shifting towards repairing damaged proteins rather than breakdown and *de novo* synthesis. Panel **(A)**: Expression of genes related to protein turnover, e.g. protein synthesis, disaggregation, (re)folding, degradation during retentostat cultivation of *L. lactis* KF147 (Ercan *et al.* 2015b). Colours represent the relative expression (log_2 scaled) compared with the initiation of retentostat cultivation where cells grow at a rate of 0.025 h^{-1}. Panel **(B)**: Schematic representation of the proposed model of protein turnover adaptation in *L. lactis* at high and near-zero growth rates. The thickness of the arrows intends to represent the relative conversion rates. At near-zero growth rates, bacteria are proposed to switch from protein degradation and *de novo* re-synthesis (steps v and vi) to disaggregation and refolding (steps iv and i) of misfolded proteins, which is supported by the induction of the ClpB-DnaKJ system as well as ribosome hibernation factor YfiA (Puri *et al.* 2014b) (see panel A). Arrow numbers indicate the different steps in protein turnover: (i) protein folding or refolding, (ii) unfolding, (iii) aggregation, (iv) disaggregation, (v) breakdown to amino acids and (vi) *de novo* synthesis.

Unfortunately, the formation of amino acid catabolism-derived flavour compounds was not determined during *L. lactis* KF147 retentostat cultivation (Ercan, Smid and Kleerebezem 2013), but it is tempting to speculate that the elevated amino acid consumption during long-term retentostat cultivation coincides with the formation of aroma components analogous to what was found for *L. lactis* FM03-V1. Notably, prolonged retentostat cultivation of *L. lactis* FM03-V1 did not lead to the production of branched-chain fatty acids that were previously reported to be produced by starvation-induced VBNC subpopulations of different *L. lactis* strains (Ganesan, Dobrowolski and Weimer 2006; Ganesan, Stuart and Weimer 2007). Although these differences agree with the notion that different strains produce different flavour compound profiles, they could also imply that regulation of amino acid catabolism is differentially regulated by the conditions used to induce growth stagnation and VBNC formation. Therefore, in order to predict the functional properties and flavour forming potential of *L. lactis* strains during cheese ripening, it is important to further elucidate the mechanisms underlying the regulation of amino acid catabolism during zero growth and/or VBNC lifestyles.

FUTURE PERSPECTIVES

This review highlights genomic and lifestyle differences of *L. lactis* inhabiting different environmental niches and explores our understanding of mechanisms involved in lifestyle adaptations when these bacteria migrate between different environments. Lactococcal gene regulation and metabolic control have been extensively studied in several model strains, mostly employing laboratory media and conditions that support relatively high growth rates. Taken together these studies indicate that the metabolism of *L. lactis* is optimised for maximal acidification rather than maximal growth rate and biomass yield. This optimisation is controlled by multiple regulatory mechanisms that are strongly intertwined, including the interplay between gene-regulation networks, carbon source import systems, glycolytic-flux, redox-balance and pyruvate dissipation pathways. Despite the recognition of the role of the multiple factors involved, our understanding of the observed switch between homolactic and mixed acid fermentation remains incomplete. Further development of advanced metabolic models that include the integration of cost-benefit calculations for the synthesis of required proteins as well as constraints in membrane transport capacities may help to achieve accurate predictions of the metabolic lifestyle of *L. lactis* under different conditions.

Some observations suggest that the history and niche adaptation of *L. lactis* strains are reflected in its general lifestyle as well as its adaptive responses during changes in environmental conditions. This is exemplified by the distinct characteristics in carbon metabolism and energy household observed for dairy and plant isolates of *L. lactis* during retentostat cultivation. These differences indicate that there is a need to expand our understanding of the regulation of gene expression and metabolism to different *L. lactis* strains in order to prevent building a knowledge base that is detailed and accurate for a model strain like *L. lactis* MG1363, but fails to represent other strains of the species. Consequently, the construction of a generic metabolic model for the species *L. lactis* would require the inclusion of some form of modelling flexibility that allows the accommodation of strain-specific physiological characteristics and its adaptation during environmental changes.

Experimental evolution has been shown to be instrumental in revealing constraints that govern lifestyle adjustments in *L. lactis*, as well as the phenotypic trade-offs that are the consequences of these constraints. Detection of such trade-offs and the exploration of the constraints that cause them could pave the way to unravelling the 'design principles' underlying the lactococcal lifestyle and its adaptability under dynamic conditions, including those that are encountered during dairy fermentation processes. Similar to what is concluded above, studies targeting such constraints and their consequential phenotypic trade-offs should preferably be expanded beyond the often-used model strains.

Recent advances highlighted the importance of dormancy states of *L. lactis* subpopulations during the ripening stages of

cheese production, where these bacteria play a critical role in flavour formation. Retentostat cultivation that aims to achieve near-zero growth rates appears to be a suitable laboratory approach to mimic the lifestyle of *L. lactis* during the ripening of cheese. Deciphering the mechanisms that regulate the adaptation to these conditions, including the control of amino acid catabolic pathways, can provide novel selection criteria for the identification of improved flavour-forming strains and/or increased control of flavour formation during cheese production. Moreover, understanding the regulatory process that controls the phenotype generalisation in zero growth cultures, which include the expansion of carbohydrate utilisation repertoires and increased stress robustness, may reveal mechanisms that are conserved in many microbes because analogous phenotypic responses are common during nutrient limitation and zero growth.

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